Alterations in the Light Transmission Through Single Lens Fibers During Calcium-Mediated Disintegrative Globulization

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Purpose. The purpose of this study was to examine changes in the light transmission through single cortical fibers isolated from the rat lens during the process of disintegrative globulization.

Methods. Single cortical fibers were isolated from adult rat lens by treatment with trypsin in a solution containing 10 mM HEPES, 10 mM EDTA, and 280 mM sucrose (pH 7.4, 300 to 310 mOsm) HEPES-EDTA-sucrose (HES) solution. The isolated fibers were illuminated by a white light source, and the light transmission through the fibers was collected by a charge-coupled device camera and quantified by digital image analysis. In some experiments, thin sections of fixed lens cells were examined using transmission electron microscopy.

Results. Enzymatic dissociation of the lens yielded elongated fibers, which, in the presence of Ringer’s solution (containing 2 mM Ca²⁺), underwent disintegrative globulization. Measurements of light transmission through elongated fibers suspended in HES solution showed maximal transmission at the center of the fiber. Exposure of the cortical fibers to Ringer’s solution led to biphasic changes in the intensity of the transmitted light. Within 5 to 10 minutes of exposure to Ringer’s solution, a general decrease in the light transmission across the long axis of the fiber was observed. Extended superfusion led to a local, apparent increase in light transmission corresponding to the formation of membrane blebs and globules. Images of disintegrated globules focused above their equator showed bright halos with dark central zones. In electron micrographs, the single fibers showed uniform electron density. No significant inhomogeneities or precipitation of intracellular crystallins was observed in globules generated from fiber cells exposed to Ringer’s solution; in addition, no high molecular weight protein aggregates were found in the globules.

Conclusions. Exposure to calcium alters the light-transmitting properties of isolated cortical fibers. The initial decrease in the average light transmittance of the fiber appears to be secondary to cell swelling and may relate to protein-based opacification. An apparent increase in light transmission through calcium-generated globules is likely because of the Becke line generated by a mismatch between the refractive index of the medium and the globule cytoplasm and accentuated by the transition from rod-shaped to spheroidal morphology. Invest Ophthalmol Vis Sci. 1997;38:586-592.

The clarity of the ocular lens has been suggested to be the result of the tight packing of variably dedifferentiated fiber cells containing a high concentration of crystallins.¹ ² The short-range interaction of crystallins in the fiber cells minimizes light scattering and promotes undistorted transmission.³ Disruption of the short-range order of the crystallins, either by syneresis⁴ or phase separation,⁵ has been shown to increase light scattering and has been suggested to contribute to opacification of the lens in some forms of experimental and clinical cataract. However, several studies of the morphology of human and animal cortical cataract show that the light-scattering centers are associated with areas containing membranous beads or globules.⁶ ⁸ The origin of such globules has not been iden-
Light Transmission Through Single Cortical Fibers

Light transmission through single cortical fibers was studied to understand the changes in light transmission during the process of calcium-mediated globulization. The formation of globules may be the underlying reason for increased light scattering because it destroys the tight packing of cortical fibers and disrupts the quasiperiodic membrane stacking essential for lens transparency. Moreover, if zones of globular deposit are indeed responsible for opacification of the lens, identification of the origin of these globules and investigations into the pathophysiological mechanisms that lead to globule formation will be useful in developing an understanding of the cellular mechanisms of cataractogenesis.

The complex architecture of the intact lens does not permit the easy access required to examine the metabolism and physiology of cortical fibers. However, we have developed methods to isolate viable, single cortical fibers to study the physiological and pathologic changes in the structure and function of cortical fibers under well-defined experimental conditions. Such preparations allow direct investigation of the cortical fibers without the complexities arising from intrafiber communications and epithelial regulation.

We observe that superfusion of isolated fibers with Ringer’s solution leads to disintegrative globulization. This process appears to be mediated by an increased influx of calcium from the source because globulization is delayed by decreasing the concentration of calcium in the external medium. Calcium-mediated disintegration could be delayed by protease inhibitors, suggesting a role for enhanced (calcium-mediated) proteolysis. Interestingly, calcium-induced disintegration does not lead to cell lysis and fragmentation but does result in the formation of resealed, discrete globules strikingly similar to those observed at the light-scattering centers of cortical cataract.

The current study was undertaken to examine the changes in light transmission of isolated, single cortical fibers during the process of calcium-mediated globulization. Our results suggest a complex series of changes in light transmission during disintegrative globulization, which may contribute to the opacification of lens during cataractogenesis.

MATERIALS AND METHODS

Sprague-Dawley rats (weight range, 200 to 250 g) were housed in accordance with the institutional guidelines and killed by a single intraperitoneal injection of sodium pentobarbital. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eyeballs were removed and immersed in Ringer’s solution (composition in mM: NaCl 150, KCl 5.4, MgCl₂ 2, CaCl₂ 2, HEPES 10, pH 7.4, adjusted with NaOH). The osmolarity of the solution was 300 to 310 mOsm.

Eyeballs were dissected, and the lenses were removed with the capsule intact. The procedure for isolation of single-lens fibers has been described. Briefly, the isolated lenses were incubated with 300–310 mOsm HEPES–EDTA–sucrose (HES) solution (composition in mM: sucrose 280, Na-EDTA 10, HEPES 10, pH 7.4) containing 0.6 mg trypsin/ml (Sigma Chemical, St. Louis, MO) for 15 minutes at 33°C, after which the solution was replenished. The lenses were reincubated in fresh solution at 32°C for 6 minutes, after which the temperature of the medium was increased gradually at a rate of 0.8°C/minute for 6 minutes and maintained at 37°C for an additional 3 minutes.

After incubation, the lenses were decapsulated, and the epithelium was removed. The lenses were rotated gently at a rate of 1 revolution/second in HES solution for 15 to 20 minutes at room temperature. The suspension was removed and plated on a coverslip and was superfused gently with HES solution to remove trypsin.

Measurement of Light Transmission

Isolated fiber cells were plated on a coverslip at the bottom of a tissue bath maintained at 35°C using an open perfusion chamber, equipped with a Peltier heating element (PDMS-I; Medical Systems, Greenvale, NY). The perfusion chamber was mounted on the headstage of an inverted microscope (Nikon [Tokyo, Japan] Diaphot 300). Cortical fibers were illuminated from above using a 6 V halogen lamp, and the transmitted light was collected by a charge-coupled device camera (model 48153100; Cuhu, San Diego, CA) connected to the side port of the microscope by a 0.7X C-mount adaptor. Images were acquired digitally using Image-1 acquisition system and software (version 4.0; Universal Imaging, West Chester, PA) and were displayed on an RGB monitor (NEC Multisyn 5FG; NEC Technologies, Boxborough, MA).

Thin-Section Electron Microscopy

Isolated lens fiber cells in small centrifuge tubes from control and experimental groups were fixed in an improved fixative containing 2.5% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.3), 50 mM L-lysine, and 1% tannic acid for 2 hours or overnight at room temperature. After a brief rinse in 0.1 M cacodylate buffer, the isolated fiber cells were centrifuged down and postfixed in 1% aqueous OsO₄ for 1 hour at room temperature, rinsed in distilled water, and stained en bloc with 0.5% uranyl acetate in 0.15 M NaCl overnight at 4°C. Isolated fiber cells were dehydrated through graded ethanol and propylene oxide and embedded in Polybed 812 resin (Polysciences, Warrington, PA). Thin sections (1 μm) were cut with a glass knife, stained with 1% toluidine blue, and examined under a light microscope. Thin sections (80 nm) were cut with a diamond knife, stained with 5% uranyl acetate and then by Reynolds’ lead citrate, and examined in a JEOL (Peabody, MA) 1200EX electron microscope.

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RESULTS

Enzymatic dissociation of the rat lens cortex yielded a significant fraction of the cell population with elongated, fiberlike morphology. These fibers excluded trypan blue, and their morphologic dimensions were comparable to those reported by us earlier. The isolated fiber cells were layered on the temperature-controlled (35°C) dish and were superfused with HES solution. The light transmitted through a fiber was collected and analyzed as described in Materials and Methods. Figure 1 shows digital images of a single fiber at various time points of superfusion. The intensity of the transmitted light is coded with pseudocolor. In HES solution, the fiber displays near uniform intensity of the transmitted light throughout its axis, with periodic gaps. These dark gaps were more prominent when the light intensity was low and when the image of the fiber was defocused slightly (as in Fig. 1A). Dark periodicities however, do not correspond to noncontiguous membrane because the fiber was morphologically intact, and it excluded trypan blue. Moreover, the average distance between the periodicities was less than the average length of cortical fibers of the lens in situ.

Measurements across the long axis of the fiber show that the intensity of the transmitted light increases from the periphery to the center. In fact, light transmission was significantly decreased at the edge of the fiber than through the HES solution, whereas light transmission through the center of the fiber was more pronounced than that through the HES solution (Fig. 1K). Line intensity profile of the image (Fig. 1F) shows near-uniform light conduction, although some differences in the intensity of the transmitted light were noted, perhaps because not all parts of the fiber presented the same transmission angle to the incident light.

As described by us earlier, superfusion of the fibers with Ringer’s solution led progressively to swelling, blebbing, and disintegration of the fiber cells into discrete globules. Figure 1 presents a series of images and line intensity profiles of the light transmitted through the fiber at various time intervals of superfusion with Ringer’s solution. Within 5 minutes of superfusion with Ringer’s solution, a marked decrease in the intensity of the transmitted light was noted throughout the long axis of the fiber (Figs. 1B, 1G). This decrease in the intensity of the transmitted light was accompanied by a slight increase in the diameter of the fiber, indicative of cell swelling. The decrease in light intensity appeared to be a general, rather than a local, phenomenon because the inhomogeneities observed in the fiber, before superfusion with Ringer’s solution, were unaltered (see Figs. 1F, 1G).

Extended superfusion with Ringer’s solution led to a progressive decrease in the intensity of the transmitted light. However, as blebs began to appear and the fiber cells started disintegrating into globules, a sharp increase in the intensity of transmitted light was observed (Fig. 1H). The increase in intensity was progressive and was maximal when the fiber completely disintegrated (t = 45 minutes) into globules (Fig. 1J). In contrast to the general decrease in the light intensity observed during the early phases of the injury (<30 minutes), the increase in light during the later part of the experiment appeared to be local and a consequence of globulization.

Figure 2 presents time-dependent changes in the intensity of light transmitted through single-fiber cells on superfusion with Ringer’s solution. For these data, the light transmitted through the entire fiber was measured, and the average pixel intensity (Fig. 2A) and light intensity integrated over the total fiber area (Fig. 2B) are shown. For measuring integrated light intensity, the area demarcated for the parent fiber was used, even though during globulization this resulted in visible gaps and discontinuities. In addition, because of the wide disparity of transmitted light (caused by differences in fiber length and diameter or by day-to-day changes in the intensity of incident light, height of solution, and so on), the values were normalized with respect to the intensity of the light transmitted at time

![Figure 1](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933197/) Changes in the intensity of transmitted light during disintegrative globulization of isolated cortical fibers. The fiber cell was isolated as described in Materials and Methods and was layered on top of a coverslip in HEPES-EDTA-sucrose (HES) solution. The fiber was superfused with Ringer’s solution, and images were acquired before (A) and after 15 (B), 25 (C), 35 (D), and 45 (E) minutes of superfusion. Corresponding color-coded histograms of transmitted light intensity are shown in the adjacent panel (F to J). (K) Distribution of transmitted light across a vertical section of an elongated fiber. The section examined is marked with a white line, and a plot of the intensity measured along this plane is shown. Note that the background intensity is 74 grey scales. The transverse distribution of the intensity of the transmitted light through a globule is shown (L). The corresponding plot shows that the intensity of transmitted light decreases from the periphery to the center. (Inset) A histogram plot of transmitted light intensity through the globule. The key for color-coding light intensity is shown below L.
zero—that is, at the beginning of the superfusion with Ringer's solution. The average light intensity showed an initial decrease, which was followed by a sharp increase after 40 minutes of superfusion. The intensity of the integrated light shows the converse.

Image analysis of single globules indicates that the intensity of light transmitted through the globules decreases from the periphery to the center (Fig. 1L). This is exactly opposite to the spatial distribution of the intensity of the light transmitted through elon-
FIGURE 2. Time-dependent changes in the intensity of transmitted light through single cortical fibers of ocular lens. Individual fibers were dissociated using trypsin as described in Materials and Methods. Average (intensity per pixel, A) or integrated (intensity through the entire fiber, B) light intensity was measured using the configuration shown in Figure 1, in HEPES-EDTA-sucrose solution (t = 0), and at the indicated times after superfusion with Ringer’s solution. A representative set of images is shown in Figure 2. Data are shown as discrete points, and the curves are the best fit of two polynomials to the data. Note that the values were normalized with respect to the intensity of the respective fiber at t = 0 (that is, before exposure to Ringer’s solution). Thus, transmittance before exposure to Ringer’s solution is expressed as 100%. The concentration of calcium in Ringer’s solution was 2 mM.

To investigate further the alterations caused by globulization, the structure of the isolated fibers was studied by transmission electron microscopy. Figure 3A shows a representative electron micrograph of a fiber cell fixed after exposure to the HES solution. Note that the single fiber cell shows uniform electron density, similar to that observed in thin sections of the intact lens cortex. Figure 3B shows two globules generated on exposure to Ringer’s solution. The cytoplasm of each globule shows no marked inhomogeneities or precipitation of intracellular crystallins. Moreover, the cytoplasm shows no indication of the formation of high molecular weight protein aggregates.

DISCUSSION

Several studies have reported that discrete membranous globules are present at the light-scattering centers of supranuclear cataracts. Nevertheless, the origin of these globules is unclear, and it is unknown how such globules are formed during cataractogenesis. Our studies with isolated fiber cells show that such globules arise spontaneously from disintegrative globulization of elongated fibers exposed to medium containing high concentration of extracellular calcium.

The process of globulization appears to be mediated by a large calcium influx and the consequent activation of proteolytic enzymes such as calpain. These results are in agreement with those of in vivo studies that suggest a critical role of calcium homeostasis.

FIGURE 3. (A) Thin-section electron micrograph showing an isolated single fiber cell after exposure to HEPES–EDTA–sucrose solution (control). The fiber cell is elongated, and the entire cytoplasm exhibits a homogenous electron density of crystalline proteins and cell matrix. (B) Transmission electron micrograph shows discrete globules formed on superfusion with the Ringer’s solution containing 2 mM Ca²⁺. The entire cytoplasm of each globule displays a homogenous electron density, without protein aggregation. Bar = 2 μm.
and the proposed association between the activation of proteases\textsuperscript{15} and cataractogenesis.

Exposure of the isolated fibers to Ringer’s solution led to an initial decrease in the average light intensity transmitted through the fiber. As indicated in Results, this decrease in transmitted light was general and was evident throughout the length of the fiber. The initial response of the fiber to Ringer’s solution is an increase in cell volume (swelling) caused by increased hydration. This is consistent with the increase in the integrated light intensity, presumably also caused by an increase in the fiber area. Increased lens hydration has been shown to be associated with the early stages of several forms of cataract.\textsuperscript{16,15} Although the mechanisms of this hydration are not well understood, it appears likely to have been caused by a net gain of Na\textsuperscript{+} and Cl\textsuperscript{−} ions by the fiber (Donnan swelling). The process of globulization appears to result from calcium-induced proteolysis (vide supra), which could involve progressive degradation of the cytoskeleton.\textsuperscript{16} This process probably also induces permeability changes so that a positive feedback loop is established, giving rise to catastrophic changes (that is, disintegration into discrete globules).

The observed decrease in average light transmission in the initial stages may result from progressive dilution of crystallins because of increased influx of water and cell swelling. In vitro experiments show that light scattering increases as the concentration of crystallins is increased up to approximately 0.12 g/ml; then it decreases.\textsuperscript{2} Concentrated (0.12 to 0.3 g/ml) solutions of crystallins transmit more light, probably because of increased short-range, liquidlike organization of the crystallins.\textsuperscript{2} Therefore, it follows that swelling or increased hydration of the lens fibers may alter crystallin organization, which may contribute to the observed decrease in transmitted light during the pre-globulization stages (Fig. 1). Changes in light transmission could be secondary to an increase in the diameter of the fiber cell that increases the light path. However, changes in the light path length alone cannot account for our observations because it appears unlikely that path length could change without altering the concentration of intracellular crystallins; this assumes that no significant de novo synthesis of crystallins occurs during globulization.

The formation of globules from fiber cells exposed to calcium-containing medium (Ringer’s solution) was associated with an increase in the average intensity of the transmitted light. The increase in the transmitted light was more pronounced than the early decrease discussed and apparently continues after the globules have detached completely from the parent fiber. The corresponding decrease in the integrated light intensity is apparently caused by the discontinuities caused by globulization. The increase in transmitted light on globulization appears paradoxical, but it probably does not result from the formation of high molecular weight aggregates in the cytoplasm because globule cytoplasm remains clear (Fig. 3). As pointed out by Bettleheim,\textsuperscript{5} protein aggregates must reach a molecular weight of more than 1 million to cause significant scattering. Proteins of such high molecular weights, however, are found primarily in the nucleus. Moreover, high molecular weight proteins are precursors of insoluble aggregates that lead to the formation of large electron-dense particles measuring as much as 500 nm in diameter.\textsuperscript{17} Clearly, no such large, electron-dense bodies are apparent in the cytoplasm of globules generated from the calcium-induced disintegration of isolated cortical fibers. In fact, the cytoplasm of the globules appears to be slightly more electron dense than the fiber (see Figs. 3A, 3B), which could contribute partially to the increase in light transmission observed during the process of globulization.

The predominant cause for the observed increase in light transmission on globulization, however, appears to be a mismatch between the refractive indices of the globules and the surrounding medium. As illustrated in Figure 4, a mismatch between the refractive index of a conducting sphere and the surrounding medium gives rise to the Becke line, which appears as a halo around the image plane when it is defocused slightly.\textsuperscript{18} The Becke line arises when a sphere is immersed in a medium with a refractive index different from that of the particle; at a certain focal plane, it appears outside the geometric edges of the sphere. As an exact focus is approached, the fringe moves inward to coincide with the edge of the sphere. Beyond this point, the bright region moves into the image of the

\begin{figure}
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\includegraphics[width=0.5\textwidth]{becke_line_diagram.png}
\caption{Formation of Becke line during measurements of light transmission through a conducting sphere observed under a microscope, when the refractive index of the medium is less than that of the sphere. Adapted from Slayter EM. The determination of refractive index. In: Optical Methods in Biology. New York: Robert E. Krieger; 1976:575–599. Reprinted by permission of John Wiley & Sons, Inc.}
\end{figure}

\textbf{Optical Methods in Biology.}


\textsuperscript{16}Donnan GB. The electrical permeability of the lens. J Physiol. 1922;54:335-346.


swelling is an early step in the process, globulization which leads to significant attenuation in light intensity. As shown in Figure 1L, a distinct halo appears around the globule forming a bright halo surrounding the edge of the globule (Fig. 1L).

Although the contribution of calcium-induced disintegration of the fiber cells to alterations in the light-transmitting properties of the lens during in vivo cataractogenesis remains to be assessed, it is expected that the formation of globules within the lens will have optical effects similar to those observed in vitro. In a normal transparent lens, there is a mismatch between the refractive indices of the intercellular space and the protein-filled fibers. However, because of quasi-periodic organization of the membranes, no scattering is expected except in the Bragg reflection, which appears within a narrow cone close to the incident light forming lenticular halos. Such halos have been observed experimentally, and their angular spacing corresponds to the width of the fiber. Nevertheless, the well-aligned cortical lattice gives rise to a diffraction pattern that maximizes light transmission by destructive interference over a large section of space. It follows that any variation in the membrane-stacking periodicity, or any disruption of the balance between form and intrinsic birefringence, will enhance optical anisotropy. However, the formation of globules may be particularly damaging because, unlike elongated fibers (or even disrupted membranes), the globules focus light locally, and this interrupts the specular reflection by tubelike fiber cells. Moreover, because swelling is an early step in the process, globulization enhances the difference in the refractive index between intracellular and extracellular space. This leads to a synergetic increase in light scattering caused by the dispersion of refractive indices and to the efficient local focusing of light by the spherical globules.

Key Words
calcium, globulization, lens fiber cells, light transmission, transmission electron microscopy

Acknowledgments
The authors thank Mr. Adell Mills for technical assistance with electron microscopy and Drs. Albert Zacarias and Masoud Motamedi for helpful discussions.

References